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PATTERN FORMATION BY OOPLASMIC SEGREGATION IN ASCIDIAN EGGS*

WILLIAM R. JEFFERY

Center for Developmental Biology, Department of Zoology, University of Texas, Austin, Texas 78712

Abstract

This article reviews the generation of cytoplasmic patterns by ooplasmic segregation in ascidian eggs. Ascidian eggs exhibit a spectacular episode of ooplasmic segregation after fertilization which finalizes the position of many different constituents with respect to the future embryonic axes. Among the elements that are rearranged by ooplasmic segregation include three different cytoplasmic regions of unique morphogenetic fate, cytoplasmic organelles, cell surface components, the cytoskeleton, and localized maternal mRNA molecules. New experimental evidence is reviewed suggesting that the reorganization of these constituents into a specific pattern in the egg is coordinated by their association in a cytoplasmic complex. The contraction of an actin filament network in this complex after fertilization is proposed to be the motive force for ooplasmic segregation. The focal point for ooplasmic segregation may be controlled by a local increase in intracellular calcium at or near the point of sperm entry.

Introduction

All of the different cell types of adult metazoans are derived during embryogenesis from a single totipotent cell—the egg. One of the most challenging problems of embryology is how this array of different cell types is generated from the egg during early development. The first differences are detectible at very early stages in the development of determinative embryos. These differences are thought to be mediated by morphogenetic determinants localized in the egg cytoplasm and unequally partitioned to the embryonic cell lineages where they affect gene expression (see Wilson, 1925; Davidson, 1976; Jeffery and Raff, 1983 for reviews). The determinants may be formed during oogenesis, but their final arrangement is often dictated by an extensive series of cytoplasmic reorganizations known as ooplasmic segregation, which occur after fertilization or during the early cleavage cycles. The role of ooplasmic segregation in organizing the distributional patterns of morphogenetic determinants makes a thorough knowledge of this event of importance to understanding how cell fates are established during early development.

After being neglected for a number of years, interest in the process of ooplasmic segregation has now been rekindled among developmental biologists and some significant discoveries have recently been made. The purpose of this article is to review ooplasmic segregation in ascidian eggs emphasizing this newly-available information. First, we will present a short introduction to ooplasmic segregation and its role in the early development of ascidian embryos. Second, we will describe the events of ooplasmic segregation in detail emphasizing the concerted involvement of cytoplasmic

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organelles, the cell surface, the cytoskeleton, and localized maternal messenger RNA (mRNA) molecules. Finally, we will consider the mechanism of ooplasmic segregation and present models that have been developed to explain how the various constituents of the egg are placed in a developmentally-significant pattern prior to the first cleavage.

Ooplasmic Segregation and its Developmental Significance

Ascidian eggs exhibit the most spectacular example of ooplasmic segregation that has yet to be described in nature. Unlike many other kinds of eggs, the visualization of ooplasmic segregation in certain ascidian eggs is greatly fascilitated by the presence of brilliantly-pigmented cytoplasmic regions. These pigmentations, which are due to the localization of specific cytoplasmic inclusions, and the fates of the regions of the egg they delineate were first noted by Conklin (1905) in *Styela partita* and have subsequently been reported in a number of different species of *Styela, Boltenia,* and *Pyura* (Table I). Other ascidian species have similar cytoplasmic inclusions in their eggs (*i.e., Ciona intestinalis;* Berg and Humphreys, 1960), but they are opaque or colorless and difficult to discern in the living egg (Reverberi, 1971).

Conklin (1905) reported five distinctly colored regions in the cytoplasm of *Styela partita* eggs, but only three of these are routinely distinguishable and can be followed with certainty during ooplasmic segregation. The following is a description of the ooplasmic organization of the unfertilized *Styela* egg. The myoplasm, which consists of yellow pigment granules and adhering mitochondria (Berg and Humphreys, 1960), encircles the entire periphery of the egg. The ectoplasm, a transparent region containing fine granular and membranous materials, which is originally derived from the sap of the germinal vesicle (GV), is present in the animal hemisphere of the egg. The endoplasm, colored gray and containing densely-packed yolk platelets, fills the remainder of the egg. Unfertilized *Boltenia* eggs consist of three cytoplasmic regions of similar composition, localization, and developmental fate as those of *Styela,* except that the myoplasm contains orange pigment granules (Berrill, 1948).

Ascidian eggs are shed as primary oocytes arrested in prophase of the first maturation division. Ooplasmic segregation and the completion of maturation is triggered

Table I

The colors of myoplasm and endoplasm in various species Styela, Boltenia, *and* Pyura

Species	Myoplasm	Endoplasm	Reference
S. partita	Yellow	Gray	Conklin (1905)
S. plicata	Yellow	White	Whittaker (1980)
S. clava	Yellow	Pink to Maroon	Jeffery (unpub.)
S. montereyensis	Light Green or Light Yellow	Dark Green	Miller cited in Abbott and Newberry (1980); Jeffery (unpub.)
B. echinata	Orange		Berrill (1948)
B. hirsuta	Orange	Colorless	Reverberi (1971)
B. villosa	Orange to Red	White	Jeffery (1982a); Abbott and Newberry (1980); Cloney (1961)
P. microcosmus	Brown	Pink-gray	Millar (1954)
P. squamulosa	Orange	Pink	Millar (1951)

by fertilization. The cytoplasmic rearrangements occur during a thirty minute period which encompasses maturation and part of the time between the completion of the second maturation division and the first cleavage. Two phases are apparent in the segregation process (Reverberi, 1961). The first phase occurs during the maturation divisions. It is probably initiated very shortly after sperm entry, when the myoplasm begins to stream down the periphery of the egg in a vegetal direction. According to Conklin (1905) the myoplasm streams toward the site of sperm entry, which is thought to be at or near the vegetal pole of the egg. The detailed features of the myoplasmic movements are ordinarily difficult to discern, even in intensely-pigmented eggs, mainly because of the opacity of the underlying yolk platelets. Extraction of segregating eggs with a non-ionic detergent (such as Triton X-100), however, dissolves the yolk platelets and has been found to highlight contrast between the myoplasmic pigment granules and adjacent areas of the egg (Fig. 1). By applying the detergent extraction procedure at various intervals during ooplasmic segregation, it has been shown that the downward movement of myoplasm is not uniform throughout the egg periphery. It appears to begin in the animal hemisphere and to progressively move toward the vegetal pole, collecting the pigment granules into a dense band at its upper margin as it proceeds (Figs. 1, 2) (Jeffery and Meier, 1983; Jeffery, unpub.). Sawada (1983) has recently

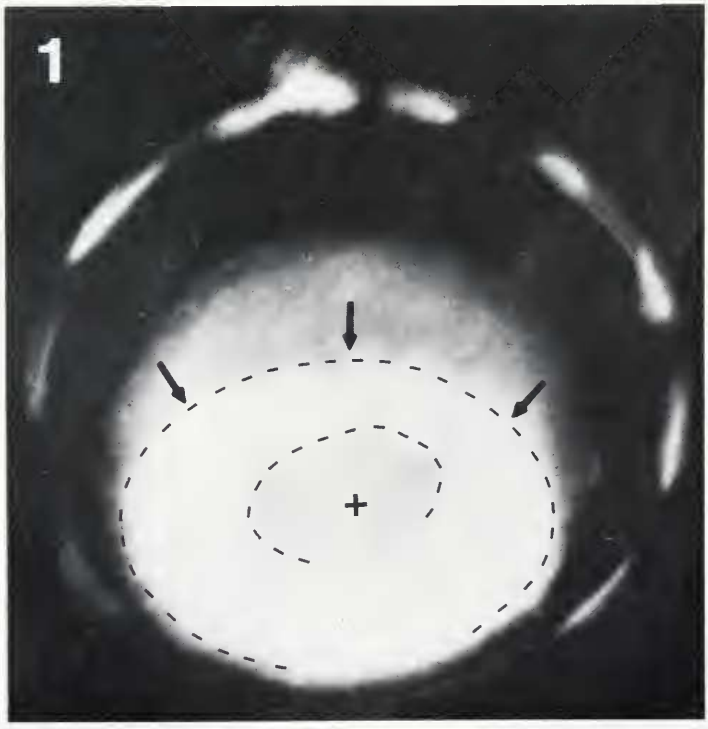


FIGURE 1. A dark field light micrograph of a *Styela plicata* egg viewed from the vegetal pole during the first phase of ooplasmic segregation. The egg was extracted with Triton X-100 as described by Jeffery and Meier (1983) to highlight the myoplasm, which can be seen as a bright ring of pigment granules in the process of streaming toward the vegetal pole. The ring of myoplasmic pigment granules is partially encircled by dotted lines. The approximate location of the vegetal pole is marked +. The arrows indicate the direction of myoplasmic streaming. The thin, bright halo around the egg is the remnant of the chorion. ×350.

shown that a thin peripheral layer of yolk platelets also streams vegetally along with the myoplasm in *C. intestinalis* eggs. As described below, however, most of the yolk moves in an animal direction during the first phase of segregation.

The marginal band of pigment granules does not reach the vegetal pole by the time the first phase of segregation is completed, and as a result the myoplasm in the polar region contains considerably fewer pigment granules and is tinted a lighter yellow than the immediately surrounding area (Fig. 2). The difference in myoplasmic coloration generated during ooplasmic segregation is maintained during later development and signifies the divergence of the larval tail muscle and mesenchyme cell lineages. The light yellow area (sometimes called the mesoplasm) eventually enters the mesenchyme cells and the surrounding darker yellow area enters the tail muscle cells (Conklin, 1905).

As the myoplasm flows into the vegetal hemisphere, most of the ectoplasm is divided into many separate islets of protoplasm in the animal hemisphere. These islets stream into the vegetal region of the egg in the wake of the myoplasm, temporarily come to rest, and then fuse again forming a transparent ectoplasmic band immediately

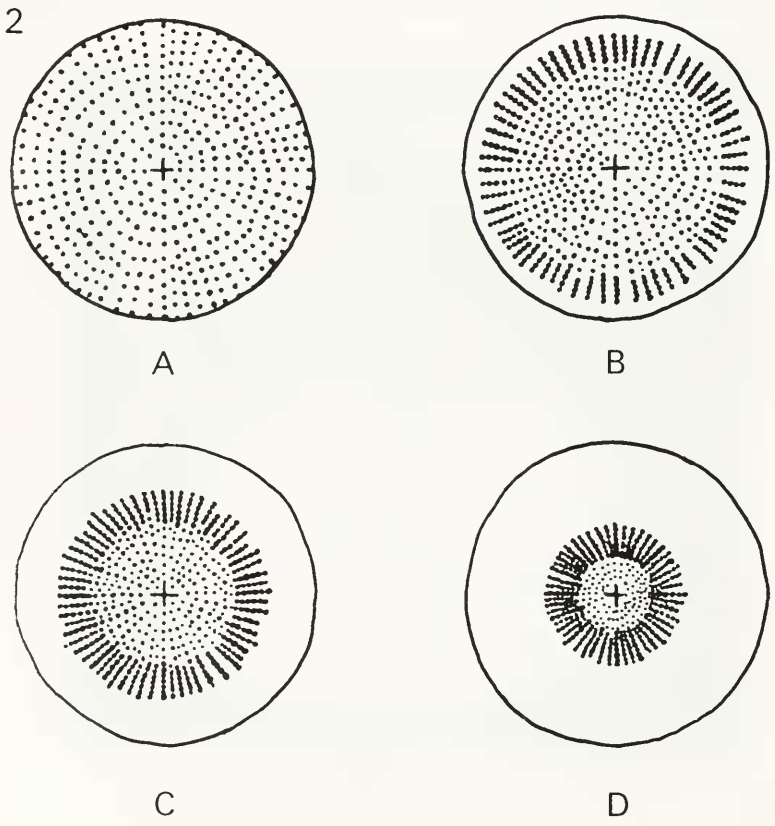


FIGURE 2. A diagrammatic representation of the movement of the myoplasmic pigment granules toward the vegetal pole of the *Styela* egg during the first phase of ooplasmic segregation. The egg surface is viewed from the vegetal pole (+). The filled circles represent individual myoplasmic pigment granules. The unfertilized condition is represented by A, while B–D represent progressive intervals during ooplasmic segregation.

above the vegetal cap of myoplasm. Most of the endoplasmic yolk platelets are displaced upwards into the animal hemisphere at the same time as the myoplasm and ectoplasm are streaming into the vegetal hemisphere. A wide protoplasmic lobe initially appears at the animal pole concomitant with the formation of the first polar body, and then at the vegetal pole at the time of the extrusion of the second polar body (Fig. 3) (Conklin, 1905; Zalokar, 1974; Sawada and Osanai, 1981). At the conclusion of the first stage of ooplasmic segregation the endoplasm, ectoplasm, and myoplasm are stratified perpendicular to the animal-vegetal axis of the egg.

The second phase of ooplasmic segregation begins at the completion of maturation. The male pronucleus forms an aster and begins to move toward the animal hemisphere, where it will eventually fuse with the female pronucleus. At this time the myoplasmic

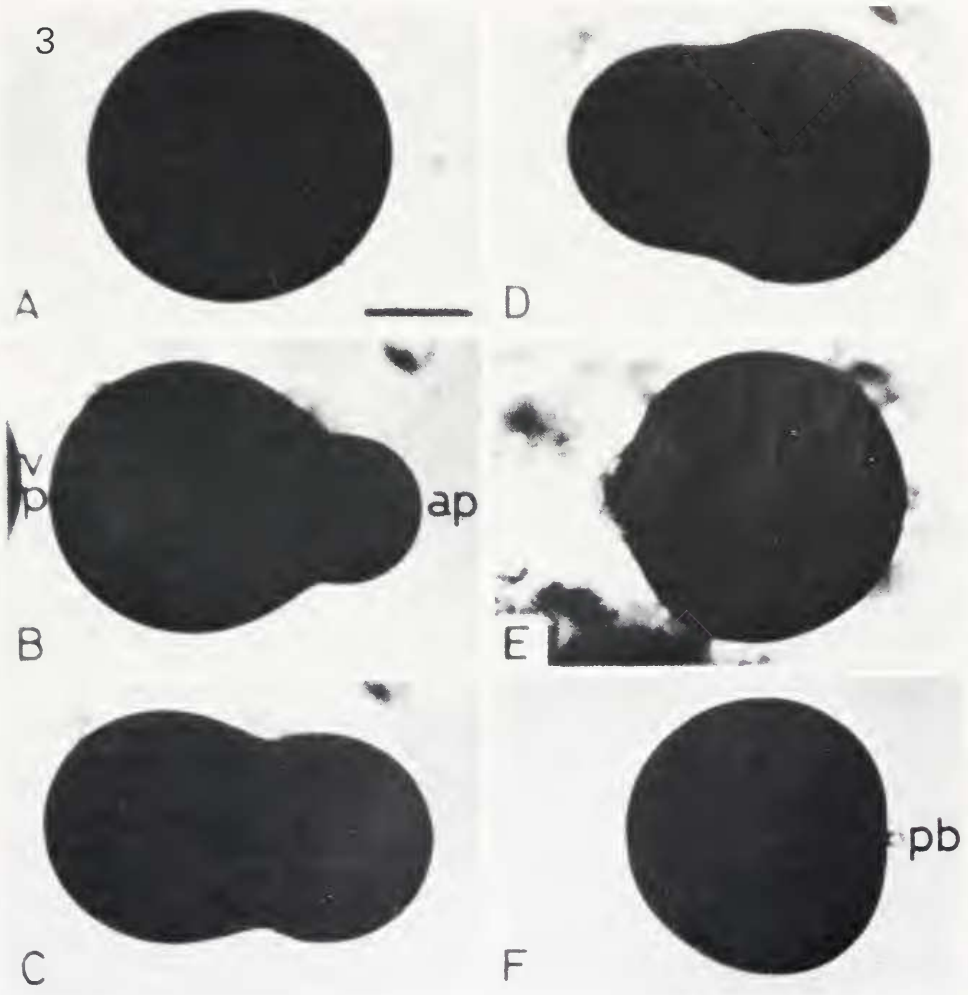


FIGURE 3. Light micrographs showing the modification of egg shape during ooplasmic segregation of dechorionated *Ciona intestinalis* eggs. A. An unfertilized egg. B–F. Successive shape changes that occur during the first phase of ooplasmic segregation. ap: Animal pole. vp: Vegetal pole. pb: Polar body. The bar in A represents 50 microns. From Sawada and Osanai (1981).

pigment granules, with their crown of ectoplasm, move upward along the vegetal periphery of the egg until they reach what will be the future posterior region of the embryo. At this location they spread out to form the myoplasmic crescent (the yellow crescent in *Styela;* Conklin, 1905). Very soon after the myoplasmic crescent is formed, the ectoplasm flows back into the animal hemisphere with the male pronucleus, where it forms a specialized cytoplasmic region around the synkaryon. At the same time, a large proportion of the endoplasmic yolk platelets return to the vegetal hemisphere. At the termination of ooplasmic segregation the ectoplasm is again located in the animal hemisphere surrounded by a thick layer of endoplasmic yolk platelets. The vegetal hemisphere, which is almost entirely filled with yolk platelets, contains the myoplasmic crescent at its posterior extremity. According to Conklin (1905), the region of the vegetal endoplasm opposite the myoplasmic crescent, which will become the anterior portion of the embryo, contains crescents of chordamesoplasm and neuroplasm.

By the conclusion of ooplasmic segregation the three major axes of the embryo are established, and ooplasmic patterns are formed in the egg which will be passed to the different cell lineages of the embryo during cleavage. The myoplasm is primarily distributed to the larval tail muscle and mesenchyme cells during embryogenesis, while the ectoplasm and endoplasm are primarily segregated into the ectodermal and endodermal cells of the embryo respectively (see Whittaker, 1979 and Nishida and Satoh, 1983 for reviews of ascidian cell lineages).

The dramatic cytoplasmic rearrangements that occur in ascidian eggs have at least two important morphogenetic consequences. First, these events appear to be responsible for setting up the final pattern of determinant distribution. Evidence for this role comes from fragmentation experiments performed on *Ciona intestinalis* and *Ascidia malaca* eggs by Reverberi (1931, 1937) and Reverberi and Ortolani (1962). They found that halves, cut in any plane of section from an unfertilized egg, each gave rise to miniature larva after fertilization. When the same cuts were made with fertilized eggs that had completed ooplasmic segregation, only the vegetal halves formed larvae. The animal fragments arrested as blastulae. Second, ooplasmic segregation seems to be responsible for determining bilateral symmetry in the embryo. As mentioned above, if unfertilized eggs are cut along the animal-vegetal axis, both halves are capable of forming complete larva. If this operation is done after the completion of ooplasmic segregation, however, complementary half-larvae are obtained from each egg half (Ortolani, 1958). These and other experiments (see Reverberi, 1961, 1971; Whittaker, 1979 for reviews) indicate that ascidian eggs are exceptionally determinative and that this property originates between the time of fertilization and the completion of ooplasmic segregation. The classical explanation of these results is that the process of ooplasmic segregation fixes the pattern of morphogenetic determinants in ways that are essential for the determination of the embryonic cell lineages. Recently, Whittaker (1980, 1982) has shown that the myoplasmic cells inherit transferable determinants that can promote the synthesis of acetylcholinesterase, an enzyme normally restricted to the tail muscle cells, in blastomeres that form non-muscle lineages.

Ooplasmic Segregation of Egg Surface Components

Since the first description of ooplasmic segregation in *Styela partita* eggs, many investigators (including Conklin, 1905) have noted that some of the test cells segregate with the myoplasm into the vegetal region of the egg forming a cellular layer above the myoplasmic crescent. Ascidian eggs are surrounded by a highly complex follicle which persists until the time of larval hatching. The follicle consists of three com-

ponents: the follicle cells, which are exposed to the outside environment, the chorion, and the test cells, which lie between the chorion and the surface of the egg (Tucker, 1942; Kessel, 1962; Jeffery, 1980). The behavior of the test cells during ooplasmic segregation implies that they may be connected to the egg surface and that components of this surface also move into the vegetal region of the egg with the myoplasm during ooplasmic segregation. There may be a practical reason for the behavior of test cells after fertilization. The test cells are known to elaborate the tail fin and associated ornamentation in the larva of some species of ascidians (Cloney and Cavey, 1982). Co-segregation with the cytoplasmic region that eventually enters the tail muscle lineage may help place the test cells in the appropriate position to perform their subsequent embryonic function. The connections between some of the test cells and elements on the egg surface may be elaborated during late oogenesis when these cells are endocytotically embedded in the cytoplasm of the developing oocyte (Tucker, 1942; Kessel, 1962). Selective staining of the test cells in *Halocynthia roretzi* has revealed long, fine filaments that extend from the test cells to the surface of the oocyte (Hirai, 1949).

Several other kinds of observations suggest that cell surface components also participate in ooplasmic segregation in concert with the myoplasm. First, supernumerary sperm, chalk granules, or carmine particles bound to the surface of dechorionated *Ciona intestinalis* eggs gradually become concentrated in the vegetal hemisphere during ooplasmic segregation (Ortolani, 1955; Sawada and Osanai, 1981). Second, stout microvilli covering the surface of unfertilized *C. intestinalis* eggs become restricted to the egg surface over the myoplasm at the completion of ooplasmic segregation (Fig. 4) (Sawada and Osanai, 1981). Finally, lectin binding sites, which are present throughout the surface of the unfertilized eggs, also co-segregate with the myoplasm after fertilization (Monroy *et al.*, 1973; O'Dell *et al.*, 1974; Ortolani *et al.*, 1977; Zalokar, 1980). It seems likely that the reorganization of the cell surface during

FIGURE 4. A transmission electron micrograph of the myoplasmic region of a *Ciona intestinalis* egg showing stout microvilli (MV) and an electron-dense layer immediately under the plasma membrane (arrows) that may represent the PML in section. From Sawada and Osanai (1981).

ooplasmic segregation can be explained by movements of extracellular elements associated with the plasma membrane. Reasonable candidates for these elements would be carbohydrate moeities associated with integral or peripheral membrane proteins.

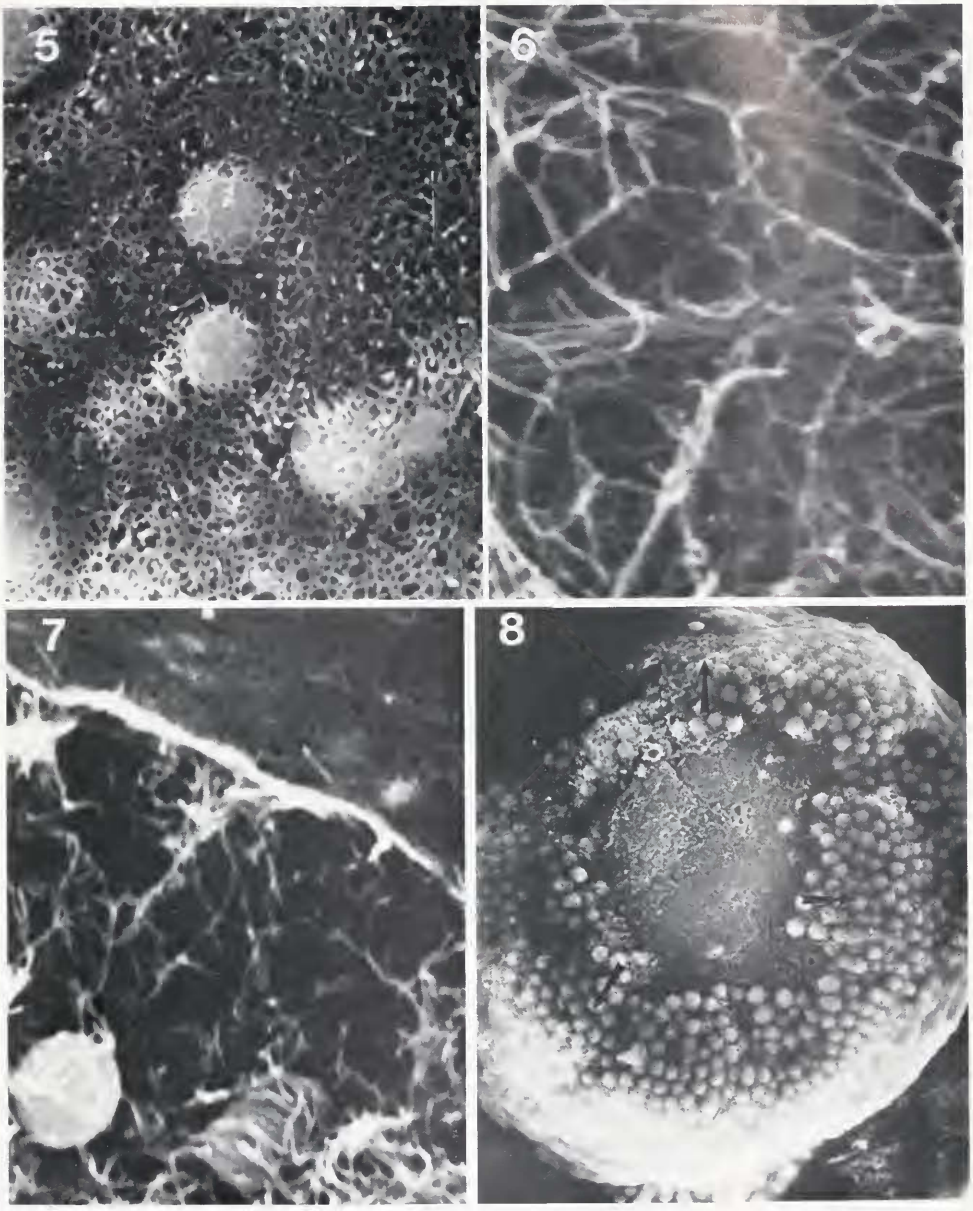
If plasma membrane proteins or other cell surface constituents translocate into the vegetal hemisphere during ooplasmic segregation and are not rapidly replaced, the lipid bilayer might be expected to be depleted of these components. In agreement with this prediction, Sawada (1983) points out that *Pyura michaelseni* eggs having recently completed segregation exhibit very fragile animal hemisphere surfaces that are the first parts of the egg to be ruptured by detergents or even by the light of a microscope lamp. This observation suggests that a local weakening of the egg surface in the animal hemisphere may be elicited by the depletion of surface components during ooplasmic segregation.

Ooplasmic Segregation of Cytoskeletal Systems

Dynamic changes occur in the cortex of many eggs during early development (see Vacquier, 1981 for review). Among the best known of these changes is the elaboration of a cortical actin cytoskeleton shortly after fertilization in sea urchin eggs (Spudich and Spudich, 1979; Wang and Taylor, 1979). There is reason to believe that cytoskeletal systems may also be elaborated in the more internal regions of sea urchin eggs after fertilization (Moon *et al.,* 1983). The participation of cortical and internal cytoskeletal elements in ooplasmic segregation has recently been demonstrated by Jeffery and Meier (1983) in *Styela* and *Boltenia* eggs. Treatment of these eggs with the non-ionic detergent Triton X-100 solubilizes most of the cellular lipids, carbohydrates, proteins, and RNA, but does not efficiently extract a group of major proteins which comprise the egg cytoskeleton. Actin and a few proteins that show similarity to intermediate filament components are among these detergent-insoluble cytoskeletal proteins. Detergent extraction also reveals an intricate cytoskeletal framework which includes some of the organelles of the egg cytoplasm. The ectoplasmic, endoplasmic, and myoplasmic regions of the egg are each represented as a special cytoskeletal domain in the detergent-insoluble residue. The different cytoskeletal domains of the egg probably represent the modern equilvalent of what early embryologists referred to as the cytoplasmic ground substance (Wilson, 1925).

The myoplasmic cytoskeletal domain has been investigated most thoroughly to date, since it is present near the egg surface throughout early development and its features can be examined by scanning electron microscopy (SEM). The myoplasmic cytoskeletal domain consists of two parts. The surface of the domain is composed of a filamentous network called the plasma membrane lamina (PML) (Fig. 5). SEM shows that the PML is arranged in a two-dimensional plane immediately below the plasma membrane of *Styela* and *Boltenia* eggs. Sawada and Osanai (1981) have seen a thin electron-dense layer below the plasma membrane in the myoplasm of *C. intestinalis* eggs by transmission electron microscopy which is likely to be analogous to the PML of *Styela* and *Boltenia* eggs (Fig. 4). Actin is a major constituent of the PML since this structure completely disappears when eggs are detergent extracted in the presence of DNase I (Jeffery and Meier, 1983), an agent known to specifically depolymerize F-actin (Hitchcock *et al.,* 1976; Raju *et al.,* 1978).

Interior to the PML the myoplasmic cytoskeletal domain consists of a three-dimensional lattice of anatomosing filaments coursed by filament bundles (Fig. 6). The deep filamentous lattice (DFL), as this structure is called, is resistant to treatment with DNase I, colchicine, or low temperatures (Jeffery and Meier, 1983; Jeffery, unpub.). Consequently, microfilaments and microtubules are unlikely to be major constituents. The DFL may consist of intermediate filaments, but this point has not



FIGURES 5–8. SEMs of the myoplasmic cytoskeletal domain of Triton X-100-extracted *Styela plicata* and *Boltenia villosa* eggs.

FIGURE 5. The PML in a *B. villosa* egg at the conclusion of the first phase of ooplasmic segregation. Note the presence of the underlying pigment granules. ×10,000.

FIGURE 6. The DFL that underlies the PML in a *S. plicata* egg. Note the filamentous lattice coursed by a filament bundle. The lattice also contains the pigment granules (not shown). ×20,000.

FIGURE 7. The PML (above) and the underlying DFL (below) viewed from the side in a *S. plicata* egg. Note the association of filaments in the lattice with both pigment granules (lower left) and the PML. ×15,000.

FIGURE 8. The myoplasmic cytoskeletal domain as seen from the animal pole of a *B. villosa* egg early during the process of ooplasmic segregation. The PML is in the process of receding into the vegetal hemisphere (arrows) ×1200. From Jeffery and Meier (1983 and unpub.) and Jeffery (1983b).

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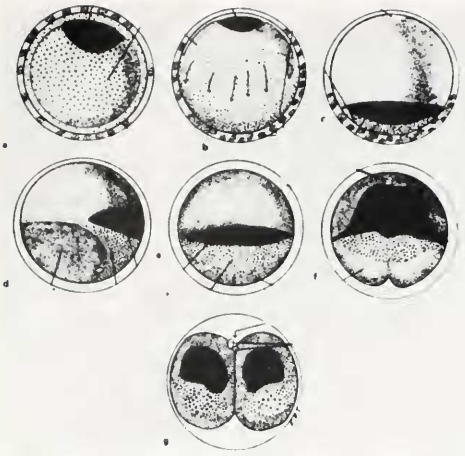
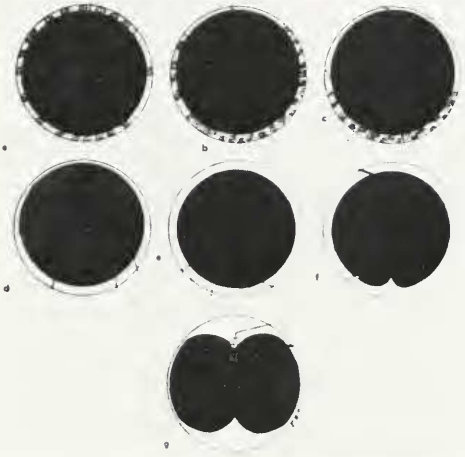
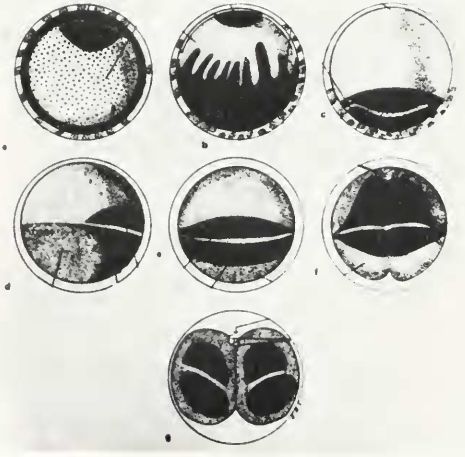
A+



Histone



Actin



been established with certainty. Jeffery and Meier (1983) have shown that filaments of the DFL are attached to the PML, to the surrounding pigment granules (these also remain after extraction), and possibly to the adjacent cytoskeletal domains (Fig. 7).

Is the myoplasmic cytoskeletal domain of ascidian eggs elaborated after fertilization, like the cortical cytoskeleton of sea urchin eggs, or is it present in unfertilized egg and segregated into the vegetal hemisphere in concert with the other myoplasmic organelles? Two lines of evidence suggest that the myoplasmic cytoskeletal domain is already present in the unfertilized eggs of *Styela, Botenia,* and *C. intestinalis.* First, SEM indicates that the PML and the underlying DFL are present throughout the cortex of unfertilized eggs. Along with the myoplasmic pigment granules, the PML and DFL progressively recede into the vegetal hemisphere after fertilization (Fig. 8). During this recession, a 2–3 fold reduction appears in the length of spaces between the filaments of the PML (Jeffery and Meier, 1983). This suggests that the PML contracts during ooplasmic segregation. Second, staining of the PML with NBD-phallacidin, a mushroom derivative that specifically reacts with F-actin filaments (Barak *et al.,* 1980), also shows that this structure is originally present throughout the periphery of unfertilized eggs and is progressively restricted to the vegetal pole region during ooplasmic segregation (Jeffery and Meier, 1983; Sawada, 1983). These results indicate that the myoplasmic cytoskeletal domain is present before fertilization and imply that it is rearranged in concert with cytoplasmic organelles and egg surface components during ooplasmic segregation.

Ooplasmic Segregation of Messenger RNA

It has been known for some time that RNA is not uniformly distributed between the various cytoplasmic regions of ascidian eggs. Histochemical studies conducted with the eggs and embryos of *C. intestinalis* (Mancuso, 1959) and *Ascidia nigra* (Cowden and Markert, 1961) showed RNA staining primarily in the ectoplasm and, to a lesser extent, in the myoplasm. The maintenance of the characteristic staining properties of these regions during ooplasmic segregation implies that RNA molecules are also subject to cytoplasmic rearrangement. The RNA type detected in these studies was undoubtedly ribosomal RNA—the most prevalent type of RNA in the egg. The segregation of ribosomal RNA is perhaps not surprising because many ribosomes are associated with intracellular membrane systems that are rearranged along with the larger cytoplasmic organelles after fertilization (Berg and Humphreys, 1960).

Histochemical staining of RNA does not shed light on the behavior of maternal mRNA, a molecule that has been considered as a candidate for a morphogenetic determinant (Whittaker, 1977; see Jeffery, 1983a for review) and that is relatively rare in the egg. The mapping of maternal mRNA distributions has recently become

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FIGURE 9. A summary diagram of maternal mRNA distribution in the three cytoplasmic regions of *Styela plicata* eggs and embryos during early development. The dark areas represent the ooplasm(s) most concentrated in the respective RNA sequences. A. Poly(A)$^+$RNA is concentrated in the ectoplasm. B. Histone mRNA is uniformly distributed between ectoplasm, myoplasm, and endoplasm. C. Actin mRNA is concentrated in the ectoplasm and myoplasm. The stages shown in frames A–C of this summary are (from left to right and top to bottom): unfertilized egg; fertilized egg during ooplasmic segregation; the end of the first phase of ooplasmic segregation, fertilized egg during second phase of ooplasmic segregation; end of the second phase of ooplasmic segregation; first cleavage; and two cell embryo. The last three stages are viewed from the future posterior region of the embryo. The data used to construct this diagram are taken from Jeffery and Capco (1978) and Jeffery *et al.* (1983). The diagram was adapted from Conklin (1905).

possible due to the advent of *in situ* hybridization methods for use with sectioned eggs and embryos (Capco and Jeffery, 1978). The results of studies on the distribution of general poly(A)$^+$RNA, actin mRNA, and histone mRNA during early development of *Styela plicata* are summarized in Figure 9. Using *in situ* hybridization with a poly(U) probe (Jeffery and Capco, 1978), maternal poly(A) [presumably poly(A)$^+$RNA] was found to be significantly enriched in the ectoplasm relative to the other cytoplasmic regions of unfertilized eggs (Fig. 9A). High levels of poly(A) persist in the ectoplasm during ooplasmic segregation, when this region migrates extensively through the egg and eventually comes to rest in the animal hemisphere. The distribution of histone and actin mRNA has been investigated during ooplasmic segregation by *in situ* hybridization with the appropriate cloned DNA probes (Jeffery *et al.*, 1983). Histone mRNA is uniformally distributed among the cytoplasmic regions of the egg, but actin mRNA is highly concentrated in the ectoplasm and the myoplasm (Fig. 9B–C). As in the case of poly(A)$^+$RNA, histone and actin mRNA segregate with the ooplasms in which they were originally localized before fertilization. These results imply that maternal mRNA molecules localized in the various cytoplasmic regions of the egg also participate in ooplasmic segregation. If maternal mRNAs do serve as morphogenetic determinants, ooplasmic segregation would seem to promote their distribution to the proper embryonic cell lineages during cleavage.

Coordination of Ooplasmic Segregation

We have seen that at least four different components of ascidian eggs; cytoplasmic organelles, cell surface elements, cytoskeletal domains, and maternal mRNA molecules, participate in ooplasmic segregation. It is possible that these components are rearranged in concert because they are associated with one another in a structural complex. In the succeeding paragraphs we will discuss the evidence for a complex of this kind, with the PML as its central element.

Morphological studies indicate that the PML is attached to the DFL (Fig. 7). The PML, through its interaction with the DFL, is also indirectly associated with the myoplasmic pigment granules, other myoplasmic organelles, and probably the ectoplasmic cytoskeletal domain (Jeffery and Meier, 1983). It seems likely (but yet to be demonstrated) that the PML may be associated with plasma membrane proteins, as are similar actin filamentous networks in somatic cells (Ben-Ze'ev *et al.*, 1979; Sheetz, 1979; Mesland *et al.*, 1981). If these membrane proteins span the lipid bilayer and interact with substances outside of the egg, the movement of test cells, surface carbohydrates, and particles that adhere to the egg surface could be coordinated with cytoskeletal and organellar rearrangements in the cytoplasm. The picture of myoplasmic organization that emerges is that of a complex containing the PML, plasma membrane proteins, and the DFL system.

The rearrangement of maternal mRNA may also be directed by an association with this complex. The interaction of mRNA with the cytoskeletal framework has been reported recently in a number of different types of eukaryotic cells (Lenk *et al.*, 1977; Cervera *et al.*, 1981; van Venrooij *et al.*, 1981; Jeffery, 1982b; Moon *et al.*, 1983). The possibility of mRNA-cytoskeletal interactions has recently been tested by subjecting sections of detergent extracted *S. plicata* eggs to *in situ* hybridization with poly (U) and cloned DNA probes (Jeffery, 1984). Most of the poly(A)$^+$RNA, histone mRNA, and actin mRNA was found in the cytoskeletal framework after detergent extraction. These molecules were also shown to be localized in the same regions of the extracted eggs as they were in intact eggs. Their localization was not altered when eggs were detergent extracted and subjected to *in situ* hybridization while the mRNA

molecules were in flux during ooplasmic segregation. These results suggest that the association of mRNA molecules with the cytoskeletal framework is responsible for their behavior during ooplasmic segregation. The nature of the egg cytoskeletal elements linked to mRNA molecules is still unknown. It seems likely that microtubule or microfilament systems (*i.e.*, the PML) are not involved, however, since the mRNA-cytoskeletal associations are insensitive to low temperature, cytochalasin B, and DNase I treatment (Jeffery, 1984). At this time the best candidate for a mRNA binding site in the myoplasmic cytoskeletal domain is the DFL.

The information at hand suggests that cell surface elements, cytoskeletal systems, myoplasmic organelles, and maternal mRNA segregate coordinately due to their association with one another in a structural complex. The movement of this complex into the vegetal pole of the egg during ooplasmic segregation may be similar to capping in somatic cells (Zalokar, 1980), where a complex of cortical actin filaments and surface ligands migrate in unison to particular regions of the cell surface (Albertini and Anderson, 1977; Toh and Hard, 1977).

Mechanism of Ooplasmic Segregation

What is the nature of the motive force required to generate the concerted movements of ooplasmic segregation? This force is likely to be generated by one of the components of the complex that moves into the vegetal hemisphere. A number of different studies suggest, however, that neither egg surface components nor internal cytoplasmic structures are responsible for ooplasmic segregation. When the lectin binding sites of *Phallusia mammillata* eggs were immobilized by attaching them to nylon sheets containing concanavalin A, the myoplasm was segregated into the vegetal region of the egg as usual after fertilization (Zalokar, 1979). This suggests that the migration of plasma membrane ligands does not drive ooplasmic segregation. When eggs were centrifuged to stratify the internal cytoplasmic components (Conklin, 1931), they still went through the shape changes characteristic of ooplasmic segregation (Sawada, 1983), they were able to move applied carmine particles into their vegetal hemispheres (Sawada, 1983), and the PML receded into the vegetal hemisphere (Fig. 10) after fertilization (Jeffery and Meier, 1984). These results suggest that the association of the DFL and attached cytoplasmic organelles with the PML is not required for ooplasmic segregation.

A number of lines of evidence suggest that the contraction of the PML drives the first phase of ooplasmic segregation. As noted above, the filamentous network of the PML has been observed by SEM to gradually recede into the vegetal hemisphere of the egg, with a concomitant shortening of its interstices, during ooplasmic segregation (Jeffery and Meier, 1983). As also pointed out earlier, it is very likely that the PML is composed of F-actin, since it disappears when eggs are extracted with detergent in the presence of DNase I (Jeffery and Meier, 1983) and it is stained by NBD-phallacidin (Jeffery and Meier, 1983; Sawada, 1983). The involvement of a microfilament system in the first phase of ooplasmic segregation is also supported by the sensitivity of this process to cytochalasin B (Zalokar, 1974; Reverberi, 1975; Sawada and Osanai, 1981). Microtubules, however, do not seem to be involved since there is no sensitivity to colchicine or vinblastine (Zalokar, 1974; Jeffery, unpub.).

Despite the availability of good evidence that the motive force for ooplasmic segregation involves a contractile system containing microfilaments, it must be admitted that filaments of this kind have never been positively identified in the cortex of ascidian eggs. It has been suggested by Reverberi (1975) that putative cortical microfilaments are too sparse to be detected in thin sections. It also seems possible that

microfilaments could be destroyed by the procedures that have been used to fix the eggs. Recently, however, Sawada (1983) has reported very sparse, 10–15 nm filaments in whole mounts of cortices isolated from *C. intestinalis* eggs that may be the elusive microfilaments.

Jeffery and Meier (1983; also see Jeffery, 1983b) and Sawada (1983) have proposed very similar models for the mechanism of ooplasmic segregation in ascidian eggs. These models are based on the contraction of an actin-containing, PML-like structure, and the association of this structure with the other elements of the egg in a structural complex that participates in ooplasmic segregation after fertilization. The Jeffery and Meier model proposes that the PML is connected to the egg plasma membrane and to more internal cytoplasmic constituents (the pigment granules, other myoplasmic organelles, the ectoplasmic cytoskeletal domain, and maternal mRNA) via the DFL. The latter connections are suggested by the observations discussed in previous sections of this article and by the direct observations of Sawada (1983) indicating that the sub-cortical myoplasm moves downward as a gelatinous mass which becomes folded, as if it were dragged by another structure (presumably the PML) (Fig. 11), during ooplasmic segregation.

According to the Jeffery and Meier model (Fig. 12), the initial event of ooplasmic segregation is a contraction of the PML. The PML is proposed to gradually shorten as ooplasmic segregation proceeds, pulling the myoplasmic domain with it on its inner surface and integral plasma membrane proteins with it on its outer surface. The accumulation of excess membrane proteins at the vegetal pole during the con-

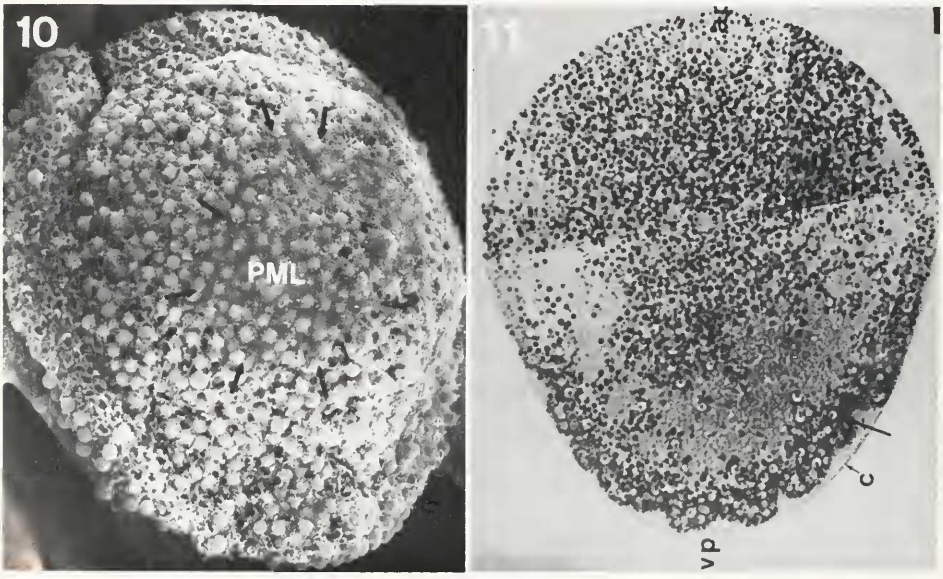

FIGURE 10. SEM of the PML of a *Boltenia villosa* egg that was centrifuged prior to fertilization to displace the DFL and associated pigment granules from the myoplasmic cytoskeletal domain and then extracted with Triton X-100 about 15 minutes after insemination. Note that the PML (outlined by arrows) has receded to a position at one end of the egg as is usual during ooplasmic segregation, but the pigment granules and associated filaments are dispersed throughout the egg. ×1200. From Jeffery and Meier (1984).

FIGURE 11. A thin section of a *Ciona intestinalis* egg undergoing the first part of ooplasmic segregation showing the buckling of the cortical cytoplasm under the plasma membrane (and presumably the PML). The bar is 10 microns. From Sawada (1983).

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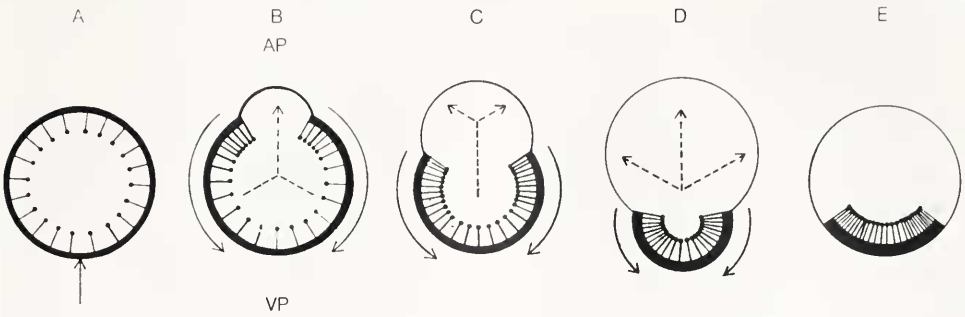

FIGURE 12. The Jeffery and Meier model for the first phase of ooplasmic segregation. A. An unfertilized egg. The vertical arrow indicates the focal point for segregation. B–D. Fertilized eggs in the process of ooplasmic segregation. The arrows represent the directions of ooplasmic movement: arrows with solid lines for the myoplasm and arrows with broken lines for the endoplasm. E. A zygote which has completed the first phase of ooplasmic segregation. In each diagram the thick egg boundaries represent parts of the plasma membrane with PML underneath, and the thin egg boundaries represent parts of the plasma membrane without the PML. The structures attached to the inside of the PML in the myoplasm represent the filaments of the DFL and its associated components, pigment granules and localized mRNA molecules of the myoplasmic cytoskeletal domain. AP, animal pole; VP, vegetal pole. From Jeffery and Meier (1983).

traction of the PML may be the reason for restriction of microvilli to this region (Fig. 4) (Sawada and Osanai, 1981). The contraction of the PML into the vegetal hemisphere is also proposed as the force that displaces most of the endoplasmic yolk platelets into the animal hemisphere. The PML is thought to form a contractile ring at its upper margin, and this ring coupled with a weakening of the plasma membrane (possibly due to the depletion of integral membrane proteins), would lead to the bulging of endoplasm in the animal hemisphere. Further tightening of the contractile ring, shortening of the PML, and endoplasmic bulging in the animal hemisphere could also make the vegetal hemisphere appear to protrude a cytoplasmic lobe (Fig. 7), as is usually seen during ooplasmic segregation (Fig. 3).

A significant question which arises from the models is whether the force generating the movement of the PML is an active, energy-dependent process, such as that based on an actomyosin system, or the recoil of a previously stretched elastic system. Treatment of ascidian eggs with ouabain, a specific inhibitor of the Na^+K^+–ATPase, or dinitrophenol, an uncoupler of oxidative phosphorylation, does not affect ooplasmic segregation, suggesting that it may not be energy dependent (Jeffery, unpub.). There is some question concerning the effect of these substances on the energetics of ascidian eggs, however, and at this time the problem of energy dependence remains open.

A way in which the Jeffery and Meier model and the Sawada model differ is that the former proposes that the complex containing the PML exists around the entire periphery of the unfertilized egg, while the latter suggests that a defect in the complex exists at the animal pole (so that the complex appears like a basket covering the lower portion of the unfertilized egg; Fig. 13A). A local break point in the PML at the animal pole of the egg must be postulated in the Jeffery and Meier model to allow the complex to contract into the vegetal hemisphere as a unit. This break point is already present in the contractile basket model of Sawada. The Sawada version is supported by observations on *C. intestinalis* (Reverberi, 1956), *Ascidiella scabra* (Dalcq and Vandebroek, 1937), and *Phallusia mammillata* (Zalokar, 1974) eggs, each of

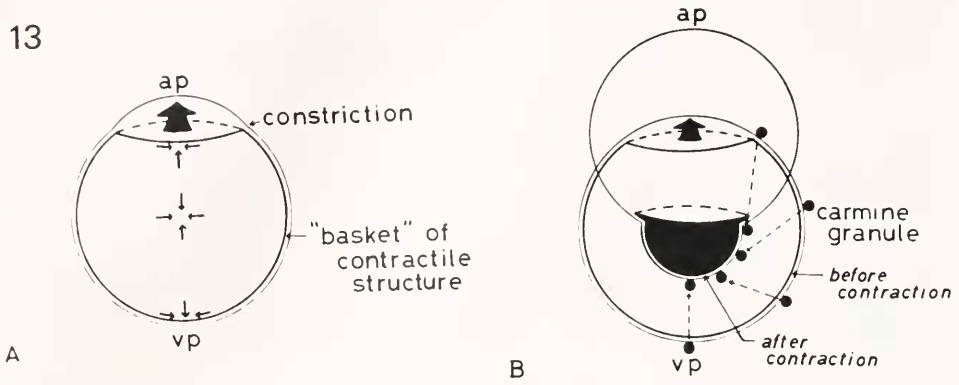

FIGURE 13. The Sawada model for the first phase of ooplasmic segregation. A. A diagram showing the contractile basket structure with a mouth near the animal pole (ap) and contracting in all directions at all points (small arrows). This contraction pushes the inner cytoplasm toward the animal pole (large arrows) making a constriction at the mouth of the basket. B. A diagram depicting the contraction of the basket into the vegetal hemisphere. The inner cytoplasm is pushed out toward the animal pole. Carmine granules are moved toward the vegetal pole (vp) as a result of the movement of surface elements dragged vegetally by the contracting basket. From Sawada (1983).

which actually show a defect in the myoplasm at the animal pole. In fact, in many instances the myoplasm is present in only the lower two-thirds of the unfertilized egg in these species, leaving a broad ectoplasmic cap in the animal hemisphere (Reverberi, 1956). Such a defect at the animal pole has not been seen by SEM or light microscopy in *Styela* or *Boltenia* eggs (Jeffery, unpub.), however, it may be too small to be detected. It is also possible that the myoplasm is thinner in the animal hemisphere than in the vegetal hemisphere of these eggs and that the myoplasmic break point is formed *de novo* after fertilization. These differences in the structure of the myoplasm could lead to variations in the manner of ooplasmic segregation between different families of ascidians.

Although the models discussed in the preceeding paragraphs provide a working hypothesis in which experiments can be devised to further understand the mechanism of the first phase of ooplasmic segregation, they do not provide insight into the mechanism of the second step in this process: the formation of the myoplasmic crescent. Unfortunately, there has been little study of the extension of the myoplasm into a crescent and the return of the ectoplasm and endoplasm to their original positions in the egg. It does seem likely, however, that the microtubular systems of the asters play an important role in these events.

CONTROL OF OOPLASMIC SEGREGATION

Cellular mechanisms must exist that regulate when and where ooplasmic segregation takes place in ascidian eggs. It is likely that such regulatory events are initiated by fertilization. A pulse of calcium, produced by an influx of ions into the fluid cytoplasm from the surrounding environment or from intracellular depots, is known to occur at the time of sperm entry in a number of different eggs (see Jaffe, 1983 for review). This transient rise in free calcium is thought to trigger the processes related to egg activation, which includes ooplasmic segregation in eggs of the brown alga *Fucus* (Jaffe and Nuccitelli, 1977). A transient rise in free calcium also appears to induce egg activation and ooplasmic segregation in ascidians, since treatment of

unfertilized *C. intestinalis* (Steinhardt *et al.*, 1974), *Ascidia malaca* (Bevan *et al.*, 1977), *Styela* (Jeffery, unpub.), and *Boltenia villosa* (Jeffery, 1982a) eggs with the divalent ionophore A23187 causes egg activation. In eggs of *Boltenia villosa,* where the process of ooplasmic segregation can be followed very precisely due to high contrast between the brilliant orange myoplasm and the white endoplasm (Jeffery, 1982a), ionophore A23187 promotes polar body formation, the streaming of myoplasm and ectoplasm into one hemisphere of the egg, egg surface deformations, and the development of a patch of orange pigment reminiscent of the myoplasmic crescent of fertilized eggs. The patch of myoplasm contains mitochondria as well as pigment granules and cannot be distinguished from a natural myoplasmic crescent by electron microscopy (Jeffery, 1982a). A cleavage furrow begins to form in many of the ionophore-treated eggs temporarily splitting the orange pigmented area, but then recedes, presumably because of the absence of a sperm aster.

It is likely that the induction of ooplasmic segregation in ascidian eggs by ionophore A23187 is the consequence of a rise in intracellular calcium, since it can be mimicked by high external concentrations of calcium or by theophylline, a substance thought to increase the intracellular calcium concentration (Bevan *et al.*, 1977). It is also inhibited by the calcium antagonist, lanthanum (Bevan *et al.*, 1977). The rise in calcium must be due to the opening of intracellular calcium depots, rather than to the entry of calcium from the surrounding environment, since the action of ionophore cannot be blocked by removing extracellular calcium from the sea water. These results suggest that a release of calcium from the intracellular depots triggers ooplasmic segregation at the time of fertilization. The calcium flux is probably mediated by the fusion of the sperm with the mature egg. The intracellular calcium depots and the cellular machinery necessary for ooplasmic segregation must be functional as early as the primary oocyte stage, however, since ovarian eggs containing intact GVs can also be induced to undergo a segregation of myoplasm by treatment with ionophore A23187 (Jeffery, 1982a).

Although a rise in intracellular calcium appears to control the timing of ooplasmic segregation, this does not explain how the directional properties of segregation are regulated. There are two major explanations for the formation of a single focal point for ooplasmic segregation. First, a rise in calcium anywhere in the cell may trigger an episode of ooplasmic segregation that is spatially directed along the animal-vegetal axis by a pre-existing determinant of egg polarity. Second, a focal point for ooplasmic segregation may be generated either toward or away from the localized region of calcium flux itself. These possibilities were tested by aligning unfertilized *Boltenia villosa* eggs against a small glass rod coated with ionophore A23187, such that only a single point on the egg surface was exposed to the antibiotic (Jeffery, 1982a). Robinson and Cone (1980) have shown that the ionophore, a substance highly insoluble in sea water, is slowly emitted from the rod forming a steep concentration gradient. The portion of the egg touching the rod was exposed to the highest concentration of ionophore and consequently would be predicted to exhibit the highest potential to release calcium from any nearby storage site. The majority of the eggs aligned against the rod in this fashion showed a segregation of orange myoplasm toward the point of contact (Fig. 14A). This was not an effect of the glass rod itself because unfertilized eggs aligned against untreated rods and then exposed to ionophore A23187 showed random foci of ooplasmic segregation with respect to the point of contact (Fig. 14B). The results suggest that the direction of ooplasmic segregation is not dictated by a pre-existing determinant of polarity in the egg, but that it is focussed toward the region of the egg cytoplasm exposed to the highest levels of calcium. It is presumed that the local rise in calcium is responsible, either directly or indirectly, for the

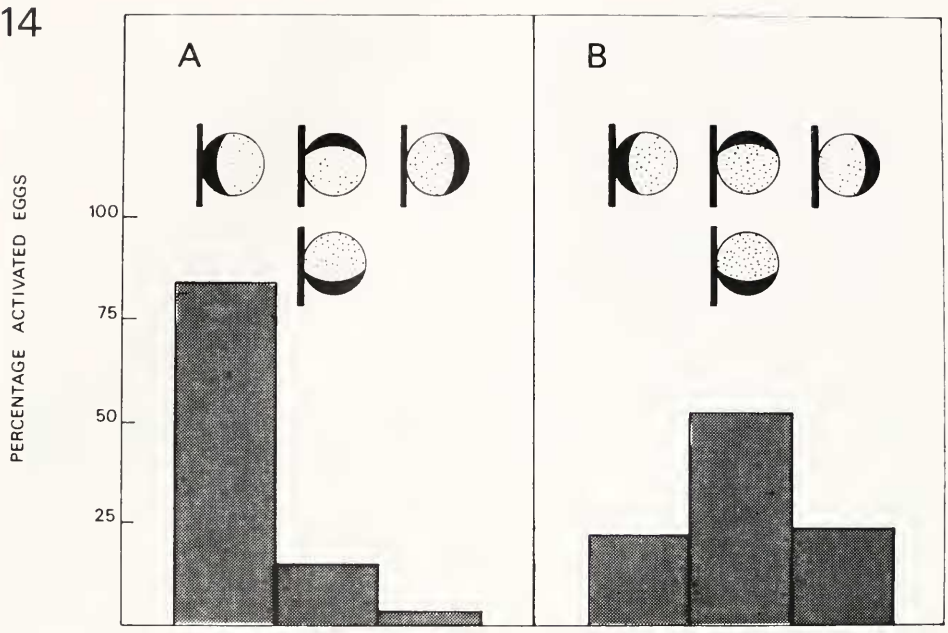

FIGURE 14. The focal points for the myoplasm in ionophore A23187-activated *Boltenia villosa* eggs that have completed ooplasmic segregation aligned against glass rods. A. The direction of myoplasmic polarization in eggs aligned against an ionophore-coated rod. Most eggs form myoplasmic caps on the side nearest the rod. B. The direction of myoplasmic polarization in eggs aligned against an untreated rod. Ionophore A23187 was present in the surrounding sea water during the experiment depicted in B. The myoplasmic caps form at random positions in the eggs with respect to the rod. From Jeffery (1983b).

contraction of the PML, which would contract toward the highest point of an intracellular calcium gradient formed in the egg. The nature of the mechanism through which calcium initiates PML contraction and the physical parameters actually responsible for the movement of the PML, however, remain to be explored.

The experiments involving a localized application of ionophore A23187 cited above also imply that many points along the circumference of the unfertilized egg are able to serve as focal points for ooplasmic segregation. Two other observations also support this idea. First, if unfertilized *Boltenia villosa* eggs are simultaneously aligned against two rods coated with ionophore A23187, many of these form double foci of ooplasmic segregation, each of which is centered near the point on the egg surface closest to the rod (Jeffery, 1982a). Second, if unfertilized *Styela* eggs are cut into as many as three fragments, each of these fragments are capable of completing ooplasmic segregation when treated with ionophore (W. R. Bates, pers. comm.). Even fragments containing less than 10% of the original volume of the egg are able to segregate myoplasm. At first these results seem to contradict the observation of Conklin (1905) that ooplasmic segregation in *Styela* eggs is focussed toward the vegetal pole. The results can be reconciled, however. The egg could be totipotent for the formation of a focal point for segregation, but under normal conditions sperm may only enter the egg and cause a local flux of calcium somewhere in the vegetal hemisphere. The problem of the spatial control of ooplasmic segregation and the subsequent fixation of cytoplasmic patterns essential for early development in ascidian eggs seems to be reduced to a single important question. Why does the sperm always enter the egg in

the vegetal hemisphere? At present there is no answer to this question. Micropyles have never been described in ascidian eggs. Indeed, the issue of a pre-determined site for sperm entry *per se* in ascidian eggs is yet to be rigorously demonstrated.

Concluding Remarks

This article has presented a review of some new information on ooplasmic segregation and its role in the generation of developmental patterns in ascidian eggs. Although we have addressed only one of the many groups of organisms that are known to exhibit ooplasmic segregation during early development, it is felt that events similar to those that occur in ascidian eggs may also take place in other species, albeit less spectacularly. For instance, a number of observations suggest that microfilaments may also be responsible for ooplasmic segregation in other kinds of eggs. Ooplasmic segregation in zebrafish eggs (Katoh, 1983), the deformation movements and rearrangement of ooplasmic constituents in *Tubifex* eggs (Shimizu, 1982), and the segregation of germ line granules in *Caenorhabditis elegans* eggs (Strome and Wood, 1983) have been shown to be sensitive to cytochalasin B. A microfilamentous network has actually been demonstrated in the cortex of *Tubifex* eggs (Shimuzu, 1982), sea urchin eggs (Spudich and Spudich, 1979; Wang and Taylor, 1979), and frog oocytes and eggs (Franke *et al.*, 1976; Gall *et al.*, 1983; Franz *et al.*, 1983). Moreover, *Xenopus laevis* eggs have recently been shown to contain a lattice of intermediate filaments interior to the cortical actin cytoskeleton (Gall *et al.*, 1983; Franz *et al.*, 1983), which is highly reminiscent of the spatial relationship between the DFL and PML in ascidian eggs.

The temporal and spatial aspects of ooplasmic segregation may also be regulated by a local calcium flux in many eggs. Ionophore A23187 promotes ooplasmic segregation in the eggs of *Fucus* (Robinson and Cone, 1980), *Tubifex* (Shimizu, 1978), and *Xenopus* (Schroeder and Strickland, 1974), and in some of these systems segregation is accompanied by the contraction of the egg cortex (Elinson, 1975; Shimuzu, 1979; Merriam and Sauterer, 1983). There are even some data, besides those already discussed for ascidians, that more than one potential focal point for ooplasmic segregation can exist in an egg. In the egg of the Medaka fish, *Oryzias laptipes,* the cortical cytoplasm normally streams toward the point of sperm entry at the animal pole and large oil droplets simultaneously flow toward the vegetal pole after fertilization. Sperm enter the egg via a micropyle in the chorion over the animal pole. If the egg is cut into two fragments, ooplasmic segregation proceeds in each fragment (Sakai, 1964). The cytoplasm streams toward the point of sperm entry in the animal fragment. If the vegetal fragment (which has no micropyle) is prick activated, however, the cortical cytoplasm usually streams toward and the oil droplets away from the site of pricking. Prick activation at two sites on the egg surface can lead to two foci of cortical streaming just as multiple sites of ionophore application can cause two myoplasmic caps to form in ascidian eggs.

Clearly, much new information on ooplasmic segregation has become available since the last review of this process was published (Costello, 1948). As more information on ooplasmic segregation is accumulated in ascidian eggs and other embryonic systems, it may be possible to develop a general hypothesis for the generation of egg patterns by cytoplasmic movements during early development.

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